PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 24/08, 33/92, A61B 5/055

A1

(11) International Publication Number: WO 91/10128

(43) International Publication Date: 11 July 1991 (11.07.91)

(21) International Application Number: PCT/US90/07622

(22) International Filing Date: 21 December 1990 (21.12.90)

(30) Priority data:
454,069 21 December 1989 (21.12.89) US
Not furnished 20 December 1990 (20.12.90) US

(71) Applicant: THE BETH ISRAEL HOSPITAL ASSOCIATION [US/US]; 330 Brookline Avenue, Boston, MA 02215 (US).

(72) Inventors: FOSSEL, Eric, T. ; McDONAGH, Jan ; 66 Priscilla Road, Chestnut Hill, MA 02167 (US).

(74) Agent: LORUSSO, Anthony, M.; Lorusso & Loud, 440 Commercial Street, Boston, MA 02109 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR PREDICTING ATHEROSCLEROTIC RISK

(57) Abstract

A method is disclosed for predicting atherosclerotic risk in a living patient using nuclear magnetic resonance. Specifically, NMR parameters for protons of high density lipoproteins are determined and compared against a corresponding value for healthy patients. Suppression of the water proton signal is employed where necessary to obtain a suitable spectrum for the non-water component protons. Individuals with high atherosclerotic risk are identified based on the mean normalized high density lipoprotein resonance amplitude of a proton nuclear magnetic resonance spectrum of a chosen body fluid, most typically one's whole blood.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

METHOD FOR PREDICTING ATHEROSCLEROTIC RISK

BACKGROUND OF THE INVENTION

Cross-Reference to Related Application

This application is a continuation-in-part of of U.S. Patent Application Serial Number 07/454,069 filed December 21, 1989.

Statement Regarding Federally Sponsored Research

Funding for work described herein was provided by the Federal Government under a grant from the Department of Health and Human Services. The Government may have certain rights in this invention.

Field of the Invention

The present invention relates to a method for predicting atherosclerotic risk in a living patient.

Prior Art

The plasma lipoproteins are complexes in which the lipids and proteins occur in a relatively fixed ratio. They carry water-insoluble lipids, such as cholesterol and cholesterol esters for eventual cellular utilization between various organs via the blood, in a form with a relatively small and constant

particle diameter and weight. While all cells require cholesterol for growth, excess accumulation of cholesterol by cells is known to result in a disease state referred to as atherosclerosis. It is also known that total serum cholesterol can be correlated with the incidence of atherosclerosis.

Human plasma lipoproteins occur in four major classes that differ in density as well as particle size as shown in the table below.

	Chylomicrons	Very low density lipoproteins (VLDL)	Low-density lipoproteins (LDL)	High-density lipoproteins (HDL)
Density, g ml^{-1}	<0.94	0.94–1.006	1.006–1.063	1.063–1.21
Flotation rate, S_f	>400	20–400	0–20	(Sediment)
Particle size, nm	75–1,000	30–50	20–22	7.5–10
Protein, % of dry weight	1–2	10	25	45–55
Triacylglycerols, % of dry weight	80–95	55–65	10	3
Phospholipids, % of dry weight	3–6	15–20	22	30
Cholesterol, free, % of dry weight	1–3	10	8	3
Cholesterol, esterified, % of dry weight	2–4	5	37	15

They are physically distinguished by their relative rates of flotation in high gravitational fields in the ultracentrifuge. All four lipoprotein classes have densities less than 1.21 g ml^{-1}, whereas the other plasma proteins, such as albumin and -globulin, have densities in the range of 1.33 to 1.35 g ml^{-1}. The characteristic flotation rates in Svedberg flotation units (S_f) of the lipoproteins are determined in an NaCl medium of density 1.063 g ml^{-1} at 26°C, in which lipoproteins float upward and simple proteins sediment.

The plasma lipoproteins contain varying proportions of protein and different typ s of lipid. The very low-density lipoproteins contain four different types of polypeptide chains having distinctive amino acid sequences. The high-density lipoproteins have two different types of polypeptide chains, of molecular weight 17,500 and 28,000. The polypeptide chains of the plasma lipoproteins are believed to be arranged on the surface of the molecules, thus conferring hydrophilic properties. However, in the very low-density lipoproteins and chylomicrons, there is insufficient protein to cover the surface; presumably the polar heads of the phospholipid components also contribute hydrophilic groups on the surface, with the nonpolar triacylglycerols in the interior. Biochemistry, Lehninger, Worth Publishers, Inc., New York, 1975, p.301.

The different lipoprotein classes contain varying amounts of cholesterol. A total serum cholesterol measurement is an average of the amount that each lipoprotein class contributes to the total serum lipoprotein.

It has long been hypothesized that the concentration of specific lipoprotein classes are predominantly responsible for the development of atherosclerosis. Studies indicate that low density lipoproteins are responsible for the accumulation of cholesterol in cells and high density lipoprot ins are responsible for removing excess cholesterol from cells.

Notwithstanding the desirability of isolating the various lipoprotein classes in blood plasma; a fast, accurate, and reproducible technique for use in clinical laboratories has not heretofore been developed. The method most often used relies on ultracentrifugation techniques, which are time consuming and expensive.

Bell et al, FEBS LETTERS, Vol. 219, no. 1, July 1987; reported single-pulse and Hahn spin-echo 500 MHz H-1 NMR spectra of human blood plasma and isolated chylomicrons, v ry low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). They made specific assignments for the resonances of individual lipoproteins in the CH_2 and CH_3 (fatty acid), and NMe_3^+ (phospholipid choline head group) regions of the spectra of plasma (0.8-1.3 and 3.25ppm, respectively). Measurements were not made on whole plasma and no attempt was made to determine the concentration of HDL or LDL or to associate them with atherosclerotic risk.

Thus, a need exists for a fast, accurate, and reproducible method to predict atherosclerotic risk in living patients.

SUMMARY OF THE INVENTION

The present invention is a method for predicting atherosclerotic risk in a living patient. In accordance with the present invention, a sample of a patient's bodily fluid is subjected to proton nuclear magnetic spectroscopy to generate a

water-suppressed proton nuclear magnetic resonance spectrum. A curve resolution procedure is performed on the methyl or methylene resonance envelope to allow classifying of the resonances as resulting from very low density lipoproteins, low density lipoproteins, high density lipoproteins and chylomicrons. The amplitude of the high density lipoprotein resonance is measured and a mean normalized value calculated. The measured amplitude is then classified into a normal or abnormal category compared to a predetermined standard for which an abnormal spectrum indicates a high atherosclerotic risk.

The VLDL, LDL, HDL and chylomicron-cholesterol values are obtained using standard purified lipoprotein preparations. Standard curves are prepared in which peak height is plotted versus concentration for the methyl or methylene resonance as well as peak area versus concentration for the methyl or methylene resonance. Test sample results are then compared to the standard curves to obtain the lipoprotein concentrations in the test sample. The triglyceride level is determined by adding the peak areas of the chylomicron and VLDL components. A risk index is determined using the peak heights and peak areas of the resolved components.

Accordingly, an object of the present invention is to provide a method for predicting atherosclerotic risk in a living patient.

Another object of the present invention is to provide the concentrations, expressed in mg/dl, of the VLDL, LDL and HDL components as well as the chylomicron and triglyceride levels.

Other objects and advantages of the invention will become apparent from the description of the drawings and the invention, which follow.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a typical 360 MHz NMR spectrum of the non-water components (water suppressed) of a plasma sample from a healthy control obtained in accordance with the present invention;

FIG. 2 is an NMR spectrum for the same plasma sample from which the spectrum of Fig. 1 was obtained, using the same equipment and pulse frequency, except without water suppression;

FIG. 3 is an expanded view of the methyl and methylene region of the Fig. 1 sample;

FIG. 4 is an NMR spectrum of the methyl and methylene regions of a plasma sample of a patient with a high atherosclerotic risk;

FIG. 5 schematically illustrates the apparatus used to perform the method of the present invention;

FIG. 6 shows the results of a study performed using the method of the present invention; and

FIG. 7 shows an NMR spectrum of the methylene region and the four lipoprotein components as obtained using the curve resolution procedure.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

At the outset, the invention is described in its broadest overall aspects with a more detailed description following.

The present invention is a method for predicting atherosclerotic risk in a living patient.

The test for high atherosclerotic risk, which is an object of the present invention, will typically be performed in vitro. The process operates on any lipid-containing body fluid, blood, or bone marrow plasma. Whole blood, serum, or plasma may be used. While the test may be performed on any such lipid-containing body fluid, work to date has focused on blood plasma and thus in a preferred embodiment of the present invention plasma is used. The test sample need not be fasting.

In accordance with the present invention, a sample of patient's whole blood or plasma is subjected to proton nuclear magnetic resonance spectroscopy to generate a nuclear magnetic

resonance spectrum. Since components of the NMR sp ctrum which have significant predictive value may be masked by other materials, such as the water signal, steps are taken to eliminate the masking, thereby producing an informative NMR spectrum.

A curve resolution procedure is performed on the methyl or methylene resonance envelope to allow classifying of the resonances as resulting from very low density lipoproteins, low density lipoproteins, high density lipoproteins and chylomicrons. FIG. 7 shows a methylene resonance NMR spectrum 1 before the curve resolution procedure and the spectrum as resolved into the four components; chylomicrons 2, VLDL 3, LDL 4 and HDL 5. The amplitude of the high density lipoprotein resonance, the amplitude of the low density lipoprotein resonance and the amplitude of the very low density lipoprotein are then measured and a mean normalized value calculated for each. The measured amplitudes are then classified into a normal or an abnormal category compared to a predetermined standard for which an abnormal spectrum indicates a high atherosclerotic risk, i.e., high HDL is a sign of low risk, high LDL is a sign of high risk.

In a preferred embodiment of the present invention, a water-suppressed proton NMR spectrum of a preprandial or postprandial plasma sample (or serum sample) is obtained and the chemical shift values of VLDL, LDL, HDL and chylomicrons in

the methyl or methylene resonance are identified using a curve resolution program. The area, line-width, and height of th VLDL, LDL, HDL and chylomicron components are then determined. The heights of the peaks are measured with a ruler or computer from the center of the baseline noise to the top of the peak.

Next, an LDL-cholesterol value is obtained using a standard purified LDL preparation. Standard curves are prepared in which peak height is plotted versus concentration for the methyl or methylene resonance as well as peak area versus concentration for the methyl or methylene resonance. Test sample results are then compared to the standard curv s to obtain the LDL-cholesterol concentration in the test sample.

An HDL-cholesterol value is then obtained using a standard purified HDL preparation. Standard curves are prepared in which peak height is plotted versus concentration for the methyl or methylene resonance as well as peak area versus concentration for the methyl or methylene resonance. Test sample results are then compared to the standard curves to obtain the HDL-cholesterol concentration in the test sample.

A VLDL-cholesterol value is then obtained using a standard purified HDL preparation. Standard curves are prepared in which peak height is plotted versus concentration for the methyl or methylene resonance as well as peak area versus

concentration for the methyl or methylene resonance. Test sample results are then compared to the standard curves to obtain the VLDL-cholesterol concentration in the test sample.

A risk index is then determined using the resolved components of either the methyl or methylene resonance using one of the following equations:

a) $$\frac{\text{Heights (LDL + HDL)}}{\text{Height HDL}}$$

b) $$\frac{\text{Areas (VLDL + HDL + LDL)}}{\text{Area HDL}}$$

c) $$\frac{\text{Heights (VLDL + HDL + LDL)}}{\text{Height HDL}}$$

d) $$\frac{\text{Areas (HDL + LDL)}}{\text{Area LDL}}$$

e) determine Height HDL relative normalized intensity using CH_3 resonances.

In a preferred embodiment, values for the risk index of 3.9 $\pm$ 4.5 at a proton frequency of 360 MHz (8.45T) or 400 MHz (9.40T) indicates a normal atherosclerotic risk or a healthy/normal individual, and a value of 11.0 $\pm$ 4.1 indicates a high atherosclerotic risk.

In addition, the concentration of VLDL, LDL, HDL chylomicrons in mg/dl is determined. Values for each lipoprotein are obtained using a standard purified lipoprotein preparation. Standard curves are prepared in which the known

lipoprotein concentration is plotted against peak area for each lipoprotein fraction using either the methyl or methylene resonance. Test sample results are then compared to the standard curves to determine the concentration in mg/dl of each lipoprotein fraction; VLDL, LDL, HDL and chylomicrons. The triglyceride concentration is then determined by adding the peak area of the chylomicron component to the peak area of the VLDL component. The peak area is measured by a standard mathematical method using a computer program to carry out the calculations.

FIG. 1 shows water suppressed proton spectrum of a healthy control, and FIG. 2 shows a proton spectrum of the same sample without water suppression. The truncated resonance line of water is denoted A in FIG. 2. The resonance lines between 2 and 3 ppm (part per million of resonance frequency) arise from the methyl and methylene groups of the lipoprotein lipids. An expanded view of this region of the proton spectrum is shown in FIG. 3 for a normal control. FIG. 4 shows an NMR spectrum of the methyl and methylene regions of a plasma sample for a patient with a high atherosclerotic risk.

In a preferred embodiment of this invention, proton NMR spectroscopy is performed on a human blood plasma sample with the water signal is suppressed. The water suppressed proton NMR spectrum obtained is dominated by resonances of plasma lipoprotein lipids. Without water suppression, these non-water resonances are virtually overwhelmed by the water. Signal

averaging allows observation of resonances associated with non-water body fluid components, at high magn tic fields, even in the presence of water resonance. However, modern NMR spectrometers can almost completely suppress the water proton resonance. The water suppressed proton NMR spectrum of plasma is essentially plasma lipoproteins and a few low molecular weight molecules. The plasma protein protons are obscured because they comprise a broad smear of unresolved resonances. The sharper resonances of the more mobile lipoprotein protons are superimposed on this broad background.

Accordingly, in its preferred embodiment, the present invention uses one of a number of conventional water suppression techniques, i.e., techniques for suppression of the water proton NMR signal. Numerous techniques have been devised to suppress the water proton NMR signal in other contexts. These may be broadly divided into two categories: (1) those that attempt avoiding excitement of the water proton signal, e.g., rapid scan correlation spectroscopy and the selective excitation technique, and (2) those that arrange for the water proton magnetization to be extremely small at the time the observed radio frequency (rf) pulse is applied, e.g., the inversion recovery technique and saturation. These and other solvent suppression techniques are described by P.J. Hore in "Solvent Suppression in Fourier Transform Nuclear Magnetic Resonance", Journal of Magnetic Resonance, 55:283-300 (1983) and the reference footnoted therein.

Although the water suppression technique is preferred when using conventional NMR apparatus due to their inability to distinguish between the signal of the solvent protons and those of the moiety or species of interest, a sufficiently sensitive apparatus would eliminate the need for water suppression altogether.

Any conventional modern NMR spectrometer may be used in the practice of the present invention. In the preferred embodiments, however, an NMR spectrometer with a magnet at constant field strength is used. The NMR signal is Fourier transformed and the mean normalized HDL resonance amplitud for proton resonances of methyl or methylene groups are the NMR parameters of interest.

FIG. 5 illustrates a nuclear magnetic resonance (NMR) spectrometer 2 which is capable of performing proton NMR spectroscopy and which is preferably, but not necessarily, of the type that suppresses the NMR signal of water. The spectrometer 2 is adapted for examination of a sample 4 which is human blood plasma contained within a test tube 6. The spectrometer 2 contains means 8 for selecting the HDL NMR resonance line in the NMR spectrum of the sample 4 and measuring the mean normalized amplitude of the resonance so selected.

The spectrometer 2 also is of conventional construction and includes, in addition to all its other structure, a means 10 for storing a value or range of values. In the preferred embodiment, the mean normalized HDL resonance amplitude is compared with a value or range of values which represents the value or range of values to be expected from normal patients, i.e., patients who are not at a high risk of developing atherosclerosis. In accordance with the invention, the spectrometer 2 also includes means 12 for classifying the measured HDL resonance amplitudes as normal or abnormal, i. ., high atherosclerotic risk, based upon the stored information. This may be done by comparison, subtraction, or any other appropriate mathematical operation.

In the preferred embodiment, the selecting and measuring means 8 is pre-adjusted to measure the mean normalized HDL resonance amplitude. This may include suppressing the water signal from the NMR spectrum of the sample 4, or may alternatively be done directly where the spectrometer 2 is sensitive enough to do so.

Typical spectrometers that can perform the method of the present invention are the Bruker AM-360 and the Bruker AM-500. Of course, others skilled in the art will know of similar equipment to perform the method of the present invention.

Correct sample preparation and execution is essential to carry out a successful measurement on plasma. Blood is collected in tubes containing 70 ul of a solution of 15% Na_2-EDTA and is maintained at 4°C until centrifugation. Plasma is separated and stored at 4°C until NMR analysis. Plasma samples are never frozen because freezing destroys lipoprotein lipid structural integrity. Samples which show any visible sign of hemolysis are excluded.

The spectra are obtained at preferably 20-22°C and most preferably at 21° C. A relatively broad range of proton frequencies may be employed, e.g., 60 MHz and higher, however, 360 MHz is the most preferred frequency. If cost is not a factor, 500 MHz may be the preferred frequency. The samples are shimmed individually on the area of the proton free induction decay until the full width at half height of the water resonance is 4 Hz of less. Careful shimming is of course an assumed component of good NMR laboratory technique.

The present invention is further illustrated by the following nonlimiting examples.

Example 1

The method of the present invention was applied to a group of 17 patients undergoing traditional lipid profile analysis. Blood was collected in non-siliconized vacutainer tubes

containing 70 ul of a solution of 15% Na_2EDTA and maintained at 4^oC until centrifugation. Plasma was separated and stored at 4^oC until NMR analysis. Plasma samples were not frozen because freezing destroys lipoprotein lipid structural integrity. Samples which showed any visible sign of hemolysis were excluded.

All spectra were obtained at 21^oC using an 8.45 T Bruker AM spectrometer operating at 360 MHz for proton (H-1) NMR. All studies were carried out in 5 mm OD sample tubes (Wilmad, Vineland, New Jersey; #507PP or #528PP). Each sample, containing 0.6 ml plasma, was shimmed individually on the area of the proton free induction decay (FID) until the full-width at half-height (FWHH) of the water resonance was 4 Hz or less. An internal quality control was found in the linewidth of the EDTA resonances. If all was well with the sample preparation and shimming, the linewidth (FWHH) of the EDTA resonances (without exponential broadening) had to be 2 Hz or less and was often between 1.0 - 1.5 Hz. In order to accomplish this, most H-1 probes require detuning to avoid radiation damping. The probe was detuned until the 90^o radio-frequency pulse became 20 msec. In the 8.45 T spectrometer, this resulted in probe detuning of about 2 MHz. The sample was spun during shimming of the Z shim coils and during data acquisition. Our H-1 spectra were acquired using presaturation to suppress water and an inversion-recovery sequence to null any lactate methyl protons present. The presaturation pulse was 4.0 sec, with a delay of about 0.8 sec between the 180^o and 90^o pulse. Eight

FIDs were signal averaged and then Fourier transformed following multiplication by an exponential resulting in 2 Hz line-broadening. The portion of the spectrum from 0.5 to 1.6 ppm was phased so that the baseline level at the edges of the plot was the same. This resulted in defective phasing of other (non-plotted) portions of the spectra.

Results of the H-1 NMR spectroscopic evaluation are shown in FIG. 6. These results were obtained by analyzing the data using the equation:

$$\text{risk value} = \frac{\text{Heights (LDL + HDL)}}{\text{Height HDL}}$$

Example 2

The method of the present invention was applied to a plasma sample. Blood was collected in non-siliconized vacutainer tubes containing 70 ul of a solution of 15% Na_2EDTA and maintained at 4^{o}C until centrifugation. Plasma was separated and stored at 4^{o}C until NMR analysis. Plasma samples were not frozen because freezing destroys lipoprotein lipid structural integrity. Samples which showed any visible sign of hemolysis were excluded.

All water-suppressed proton NMR spectra were obtained at 16-18^{o}C using an 8.45 T Bruker AM spectrometer operating at 360 MHz with zero-filling to 16K or 32K data points. All studies were carried out in 5 mm OD sample tubes (Wilmad, Vineland, New

Jersey; #507PP or #528PP). Each sample, containing 0.6 ml plasma, was shimmed individually on the area of the proton free induction decay (FID) until the full-width at half-height (FWHH) of the water resonance was 4 Hz or less.

An internal quality control was found in the linewidth of the EDTA resonances. If all was well with the sample preparation and shimming, the linewidth (FWHH) of the EDTA resonances (without exponential broadening) had to be 2 Hz or less and was often between 1.0 - 1.5 Hz. In order to accomplish this, most H-1 probes require detuning to avoid radiation damping. The probe was detuned until the 90^{o} radio-frequency pulse became 20 msec. In the 8.45 T spectrometer, this resulted in probe detuning of about 2 MHz. The sample was spun during shimming of the Z shim coils and during data acquisition. Our H-1 spectra were acquired using presaturation to suppress water and an inversion-recovery sequence to null any lactate methyl protons present. The presaturation pulse was 4.0 sec, with a delay of about 0.8 sec between the 180^{o} and 90^{o} pulse.

Eight FIDs were signal averaged and then Fourier transformed following multiplication by an exponential resulting in 2 Hz line-broadening. The portion of the spectrum from 0.5 to 1.6 ppm was phased so that the baseline level at the edges of the plot was the same. This resulted in defective phasing of other (non-plotted) portions of the spectra.

The chemical shift values of VLDL, LDL, HDL and chylomicrons in the methyl and methylene resonances were identified. A curve resolution program, such as NMRtwo from NMRi of Syracuse, NY, was used to resolve the component residues using initial spectrum and chemical shift values of the methyl and methylene resonances of the VLDL, LDL, HDL and chylomicrons. The area, line-width, and height of each resolved component was obtained.

Next, an LDL-cholesterol value was obtained using a standard purified LDL preparation. Standard curves were prepared in which peak height was plotted versus concentration for the methyl and methylene resonances as well as peak area versus concentration for the methyl and methylene resonances. Test sample results were then compared to the standard curves to obtain the LDL-cholesterol concentration in the test sample.

An HDL-cholesterol value was then obtained using a standard purified HDL preparation. Standard curves were prepared in which peak height was plotted versus concentration for the methyl and methylene resonances as well as peak area versus concentration for the methyl and methylene resonances. Test sample results were then compared to the standard curves to obtain the HDL-cholesterol concentration in the test sample.

A VLDL-cholesterol value was obtained using a standard purified HDL preparation. Standard curves were prepared in which peak height is plotted versus concentration for the

methyl or methylene resonance as well as peak area versus concentration for the methyl or methylene resonance. Test sample results were then compared to the standard curves to obtain the VLDL-cholesterol concentration in the test sampl .

A risk index was then determined using the resolved components of either the methyl or methylene resonance using one of the following equations:

a) $\dfrac{\text{Heights (LDL + HDL)}}{\text{Height HDL}}$

b) $\dfrac{\text{Areas (VLDL + HDL + LDL)}}{\text{Area HDL}}$

c) $\dfrac{\text{Heights (VLDL + HDL + LDL)}}{\text{Height HDL}}$

d) $\dfrac{\text{Area (HDL + LDL)}}{\text{Area LDL}}$

e) determine Height HDL relative normalized intensity using CH_3 resonances.

The concentrations of VLDL, LDL, HDL and chylomicrons, expressed in mg/dl, was determined by measuring the peak area of each resolved component using the methylene or methyl resonance spectrum. Standard curves were prepared where known concentrations of the lipoprotein fractions were plotted against the peak area and these were compared to the test results to determine the concentration of VLDL, LDL, HDL and chylomicrons in the test sample. The triglyceride concentration was then determined by adding the peak area of the chylomicron

component to the peak area of the VLDL component. The peak area was measured by a standard mathematical method using a computer program to carry out the calculations.

The invention may be embodied in other specified forms without departing from the spirit of essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range or equivalency of the claims are therefore intended to be embraced therein.

What is claimed is:.

1. A method for predicting atherosclerotic risk in a living patient, comprising the following steps:

(a) subjecting a non-fasting blood component sample from a patient to be tested to proton nuclear magnetic resonance spectroscopy to generate a nuclear magnetic resonance spectrum from which undesirable signals have been suppressed;

(b) performing a curve resolution procedure on the methylene resonance envelope;

(c) classifying the resonances as resulting from VLDL, LDL, HDL and chylomicrons;

(d) measuring the high density lipoprotein resonance amplitude; and

(e) classifying the amplitude measured into either a category of normal amplitude or intc a category of an abnormal amplitude as compared to a predetermined standard for which abnormal amplitudes indicate a high atherosclerotic risk.

2. The method of claim 1 wherein the curve resolution procedure in step (b) is performed on the methyl resonance envelope.

3. The method of claim 1, wherein said subjecting step of proton NMR spectroscopy (a) includes suppressing the water signal.

4. The method of claim 1, wherein said subjecting step of proton NMR spectroscopy (a) comprises obtaining a blood sampl from the patient, removing red cells therefrom, and subjecting plasma in the blood sample to nuclear magnetic resonance spectroscopy.

5. The method of claim 1, wherein the proton resonance frequency is above 60 MHz.

6. The method of claim 1 wherein said measuring step (d) comprises the step of computing the mathematical means of the normalized HDL resonance amplitude measured.

7. The method of claim 1 wherein said proton nuclear magnetic resonance is obtained at $21^{o}C$.

8. The method of claim 1 wherein the blood component sample in step (a) is a fasting sample.

9. The method of claim 4 wherein the proton resonance frequency is equal to or above 360 MHz.

10. The method of claim 6 wherein a mean normalized high density lipoprotein resonance amplitude of 3.9 ± 4.5 indicates a normal atherosclerotic risk.

11. The method of claim 6 wherein a mean normalized high density lipoprotein resonance amplitude of 11.0 ± 4.1 indicates a high atherosclerotic risk.

12. A method for predicting atherosclerotic risk comprising:

(a) obtaining a water-suppressed proton NMR spectrum of a non-fasting plasma or serum sample;

(b) identifying the chemical shift values of VLDL, LDL, HDL and chylomicrons in the methyl resonance;

(c) performing a curve resolution procedure to resolve the component residues using the initial spectrum and the chemical shift information of step (b);

(d) obtaining the area, line-width, and height of the VLDL, LDL, HDL and chylomicron components;

(e) preparing a standard curve using a standard purified LDL preparation in which peak height is plotted versus concentration for the methyl resonance;

(f) preparing a standard curve using a standard purified LDL preparation in which peak area is plotted versus concentration for the methyl resonance;

(g) comparing said plasma or serum sample results of step (a) with the standard curves obtained in steps (e) and (f) to obtain an LDL-cholesterol concentration;

(h) preparing a standard curve using a standard purified HDL preparation in which peak height is plotted versus concentration for the methyl resonance;

(i) preparing a standard curve using a standard purified HDL preparation in which peak area is plotted versus concentration for the methyl resonance;

(j) comparing said plasma or serum sample results of step (a) with the standard curves obtained in steps (h) and (i) to obtain an HDL-cholesterol concentration;

(k) preparing a standard curve using a standard purified VLDL preparation in which peak height is plotted versus concentration for the methyl resonance;

(l) preparing a standard curve using a standard purified VLDL preparation in which peak area is plotted versus concentration for the methyl resonance;

(m) comparing said plasma or serum sample results of step (a) with the standard curves obtained in steps (k) and (l) to obtain an VLDL-cholesterol concentration; and

(n) means for obtaining a risk index from the resolved components of the methyl resonance.

13. The method of claim 12 wherein the plasma or serum sample in step (a) is a fasting sample.

14. The method of claim 12 wherein steps (b), (e), (f), (h), (i), (k), (l) and (n) are carried out using the methylene resonance.

15. The method of claim 12 wherein said means for obtaining a risk index further comprises completing the following equation:

$$\frac{\text{Heights (LDL + HDL)}}{\text{Height HDL}}$$

16. The method of claim 12 wherein said means for obtaining a risk index further comprises completing the following equation:

$$\frac{\text{Areas (VLDL + HDL + LDL)}}{\text{Area HDL}}$$

17. The method of claim 12 wherein said means for obtaining a risk index further comprises completing the following equation:

$$\frac{\text{Heights (VLDL + HDL + LDL)}}{\text{Height HDL}}$$

18. The method of claim 12 wherein said means for obtaining a risk index further comprises completing the following equation:

$$\frac{\text{Areas (HDL + LDL)}}{\text{Area LDL}}$$

19. The method of claim 12 wherein said means for obtaining a risk index further comprises obtaining the Height HDL relative normalized intensity using CH_3 resonances.

20. The method of claim 12 wherein said water-suppressed proton NMR spectrum of a plasma or serum sample is obtained at 16-18^{o}C.

21. A method of measuring lipoprotein concentrations comprising;

(a) obtaining a water-suppressed proton NMR spectrum of a non-fasting plasma or serum sample;

(b) identifying the chemical shift values of VLDL, LDL, HDL and chylomicrons in the methyl resonanc ;

(c) performing a curve resolution procedure to resolve the component residues using the initial spectrum and the chemical shift information of step (b);

(d) obtaining the area of the VLDL, LDL, HDL and chylomicron components;

(e) preparing a standard curve using a standard purified LDL preparation in which peak area is plotted versus concentration for the methyl resonance;

(f) comparing said plasma or serum sample results of step (a) with the standard curve obtained in step (e) to obtain an LDL-cholesterol concentration expressed in mg/dl;

(g) preparing a standard curve using a standard purified HDL preparation in which peak area is plotted versus concentration for the methyl resonance;

(h) comparing said plasma or serum sample results of step (a) with the standard curve obtained in step (g) to obtain an HDL-cholesterol concentration expressed in mg/dl;

(i) preparing a standard curve using a standard purified VLDL preparation in which peak area is plotted versus concentration for the methyl resonance;

(j) comparing said plasma or serum sample results of step (a) with the standard curve obtained in step (i) to obtain an VLDL-cholesterol concentration; and

(k) adding the areas of the VLDL and chylomicron components obtained in step (d) to determine the triglyceride concentration expressed in mg/dl.

22. The method of claim 21 wherein steps (b), (e), (g) and (i) are carried out using the methylene resonance.

23. The method of claim 21 wherein the plasma or serum sample in step (a) is a fasting sample.

FIG. 1

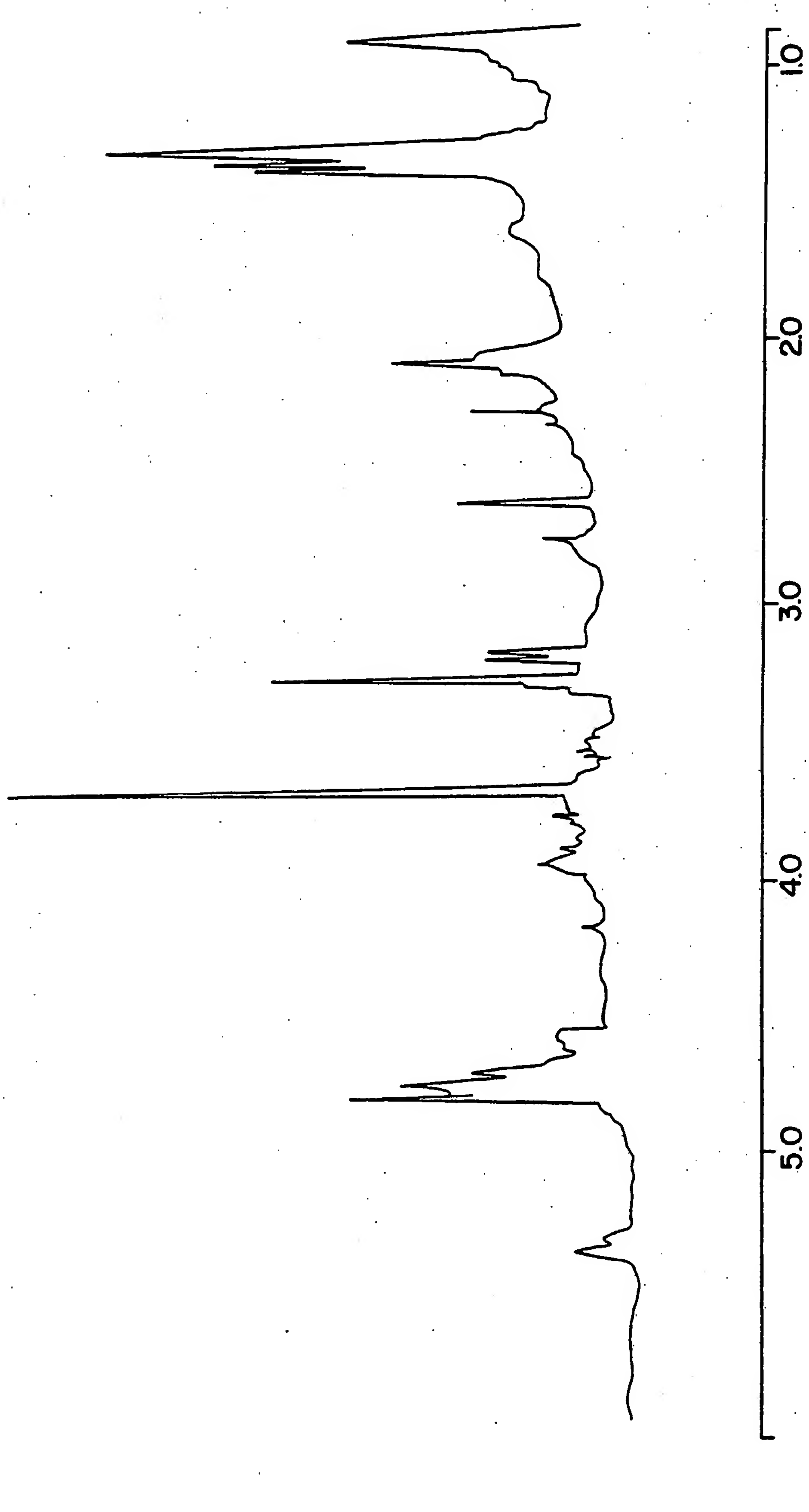
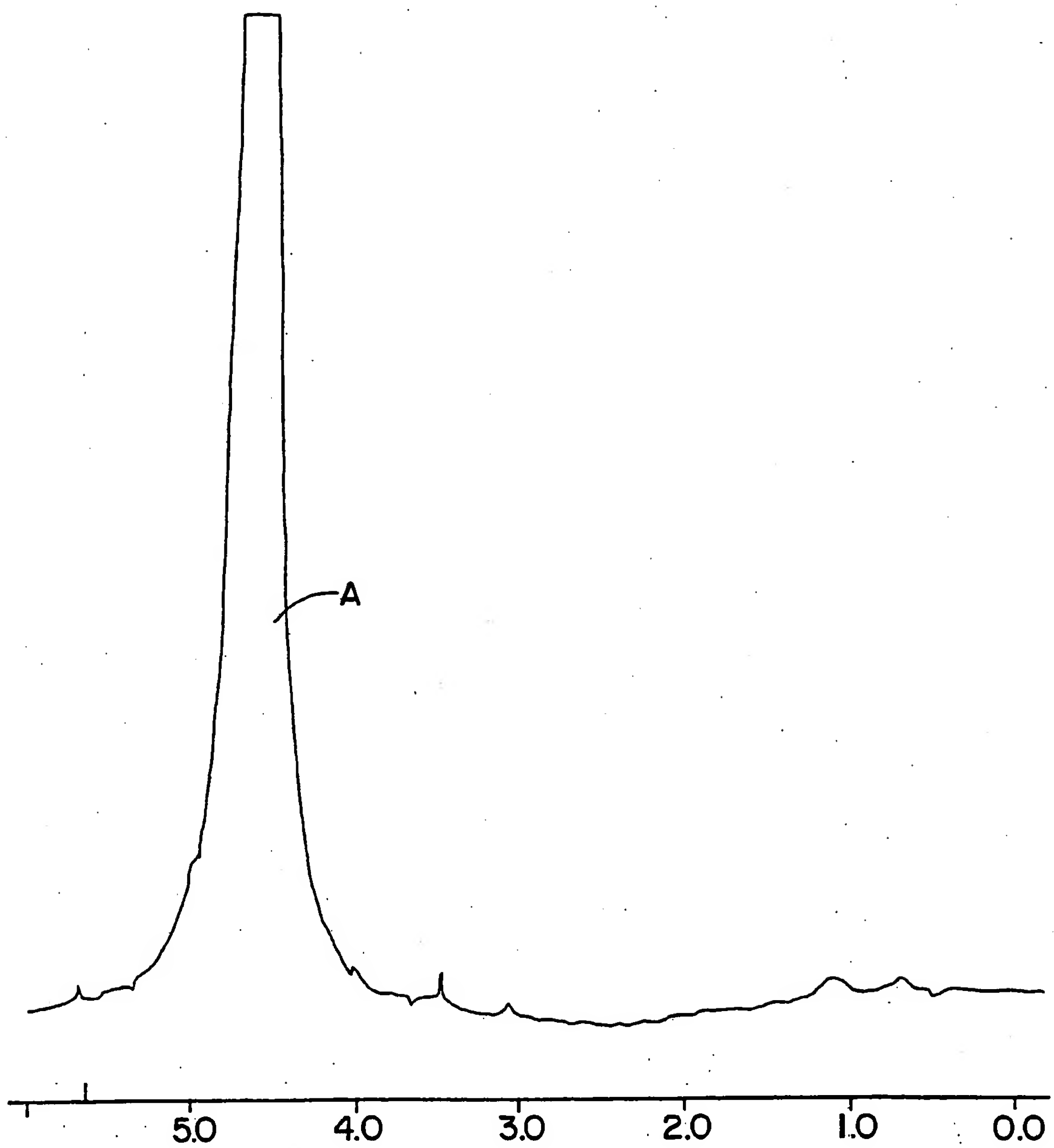


FIG. 2



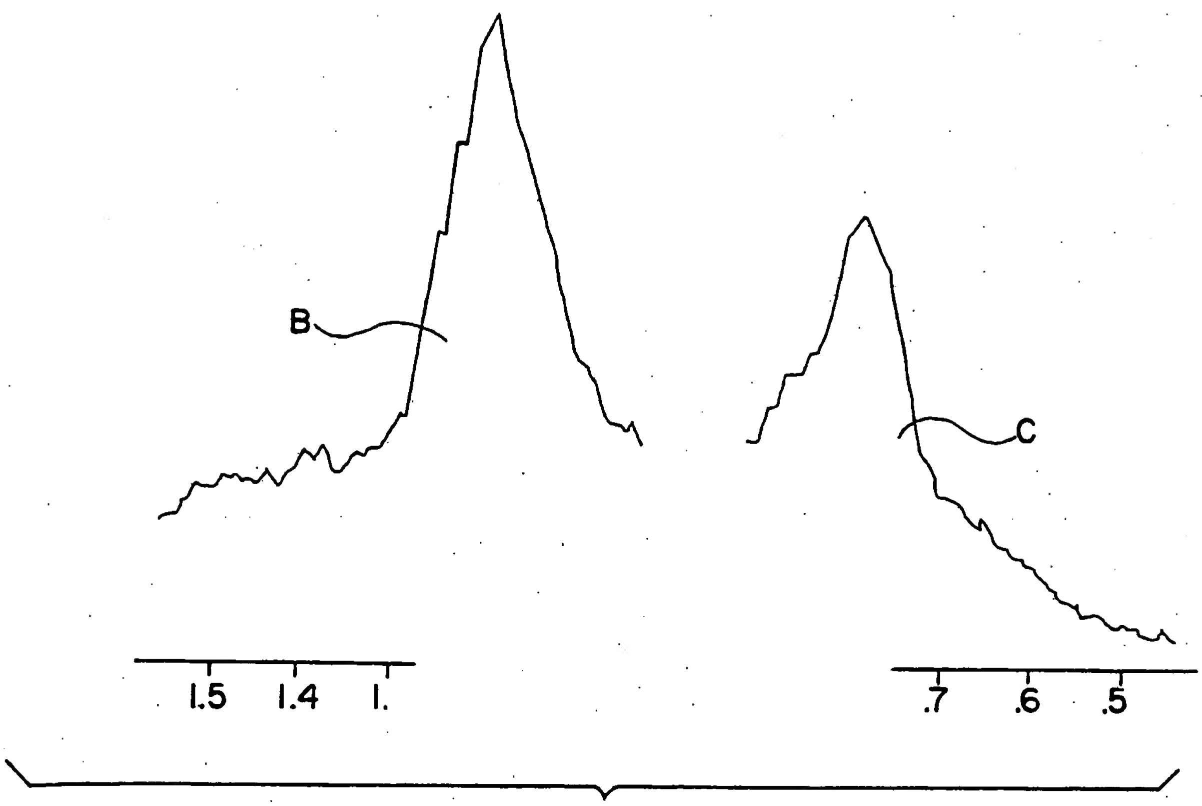


FIG. 3

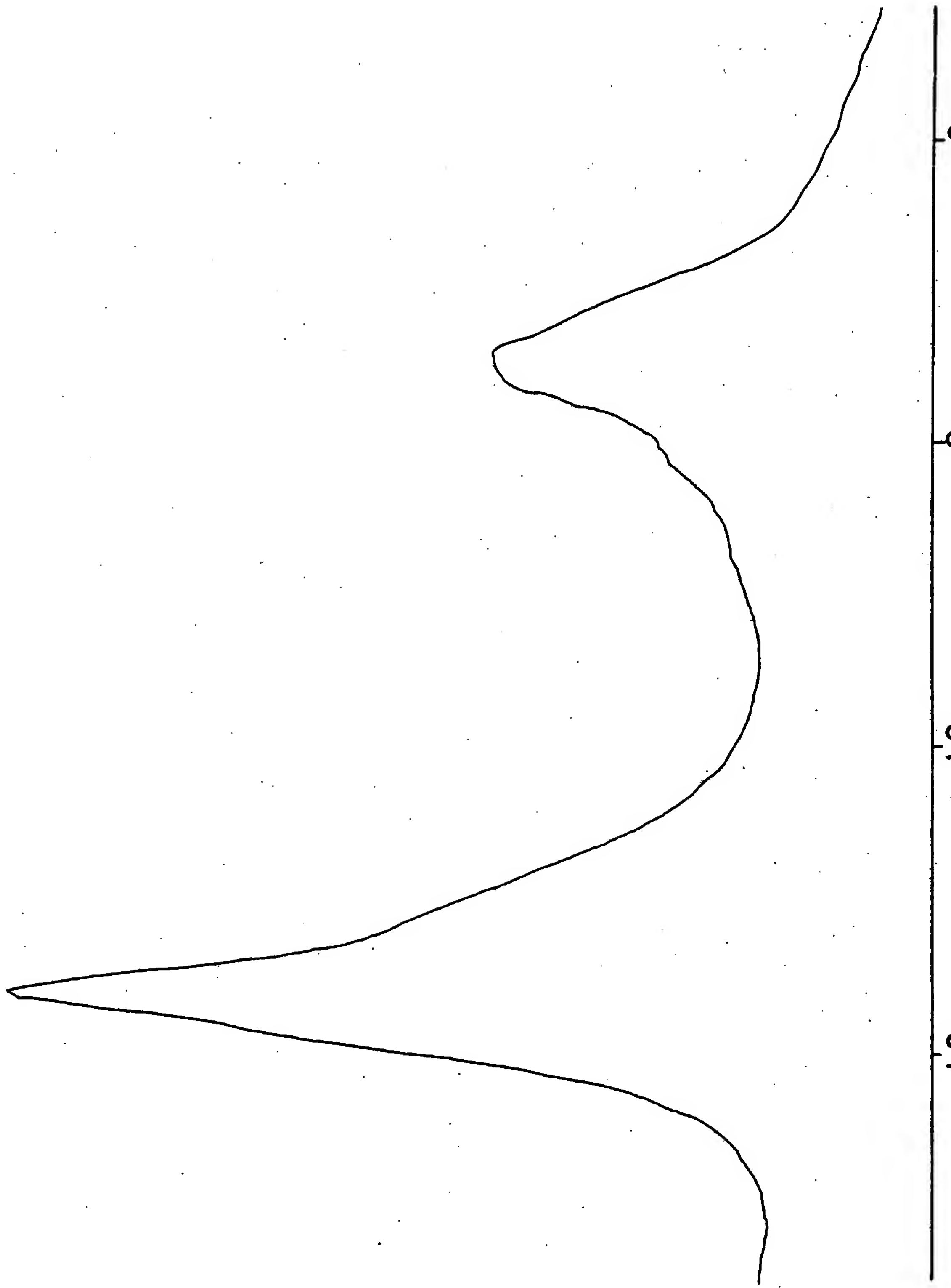


FIG. 4

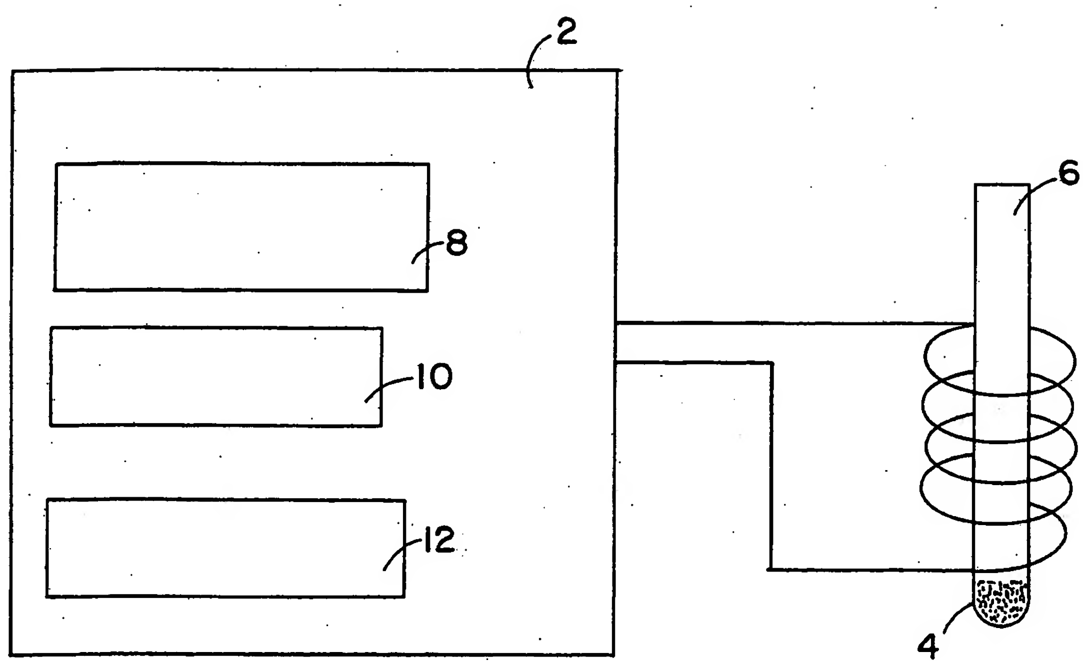


FIG. 5

FIG. 6

A		B	
2.0	3.5	4.7	8.6
2.2	3.7	4.9	8.6
2.2	3.7	4.9	8.8
2.3	3.7	4.9	8.9
2.4	3.7	4.9	9.7
2.5	3.7	4.9	10.5
2.6	4.0	5.2	10.7
2.6	4.0	5.5	10.7
2.7	4.0	5.6	12.5
2.9	4.0	5.6	
2.9	4.0	5.6	
3.0	4.0	5.8	
3.0	4.2	6.0	
3.0	4.3	6.0	
3.0	4.3	6.1	
3.0	4.4	6.2	
3.1	4.4	6.3	
3.2	4.7	6.3	
3.2	4.9	6.6	
3.2	10.8	7.2	
3.4		8.1	
3.4		8.1	
3.5			
3.5			

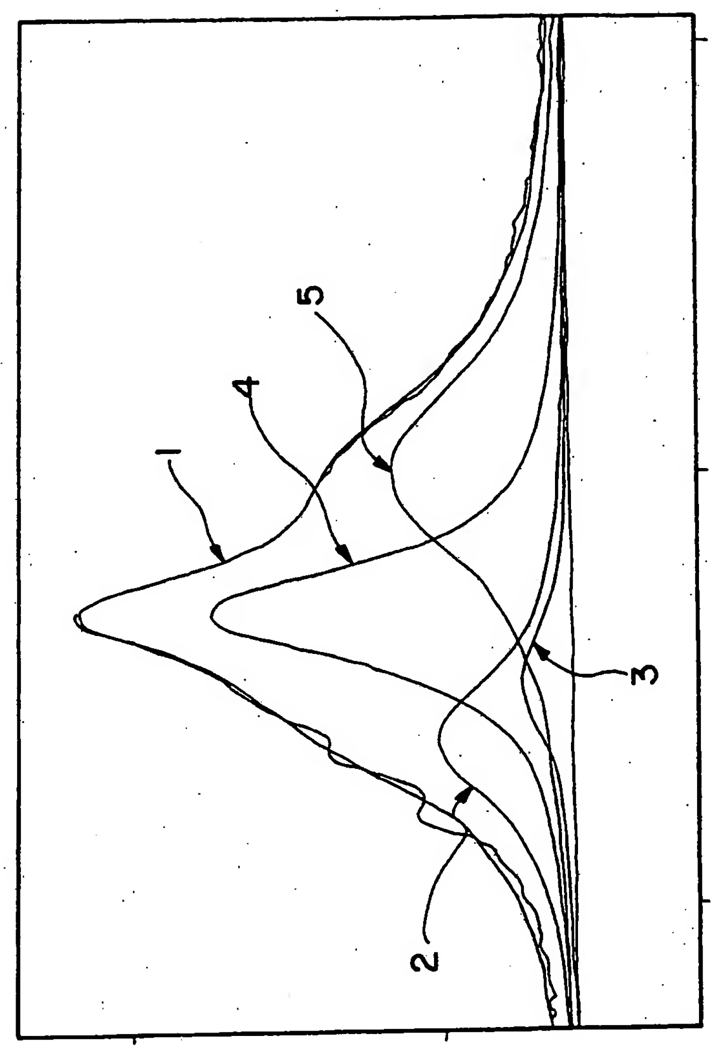


FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No I/US90/07622

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): GOIN 24/08, 33/92; A61B 5/055
U.S. Cl.: 436/71,173,811; 128/653A

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	436/57,63,71,173,811; 128/653A

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X,P	US,A, 4,940,055 (BROWN) 10 JULY 1990, see entire disclosure.	1-23
A	US,A, 4,200,588 (LEDNICER) 29 APRIL 1980, see entire disclosure.	1-23

* Special categories of cited documents: [15]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
04 APRIL 1990	09 MAY 1991
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Kimberly A. Trautman KIMBERLY A. TRAUTMAN